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(54) Title: A PROCESS OF INHIBITING NON-NEOPLASTIC PATHOLOGICAL CELL PROLIFERATION

(57) Abstract

The present invention relates to the use of gene transfer to inhibit non-neoplastic pathological cell proliferation. An eukaryotic expression vector comprising a polynucleotide sequence that codes for a dominant-negative cell cycle regulatory protein is delivered to a proliferating cell. Delivery is accomplished by infusing the expression vector into an artery that perfuses or contains the proliferating cell.

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A Process Of Inhibiting Non-Neoplastic Pathological Cell Proliferation

Description

Related Ownership Rights

Research for the information disclosed herein was supported in part by the National Institute of Health. The United States government may own certain rights to the invention disclosed herein.

Technical Field of the Invention

The present invention relates to a process of inhibiting non-neoplastic pathological cell proliferation in a cell *in vivo*. A process of the present invention involves transducing a cell with an eukaryotic expression vector comprising a polynucleotide that encodes a dominant-negative cell cycle regulatory protein.

Background of the Invention

Somatic gene therapy can be defined as the ability to program the expression of foreign genes in non-germ line (i.e., non-sperm and egg) cells of an animal. Methods of somatic gene therapy can be divided into two categories. *ex vivo* gene therapy involving the removal of cells from a host organism, transfection of a foreign gene into those cells, and re-implantation or transplantation of the transformed or transgenic cells back into a recipient host. In contrast, *in vivo* gene therapy involves transfection of a foreign gene directly into cells of a recipient host without the need for prior removal of those cells from the host.

The utility of somatic gene therapy for human subjects is dependent upon a number of factors. First, the transfection method must be efficient. Second, expression of the foreign gene should be localized to specific target tissues. Third, a given transfection process should be associated with a minimal risk of mutating the host cells and of causing a persistent infection of the host organism.

Several possible strategies to introduce genes into tissues of the body have been employed in the past (Stratford-Perricaudet et al., 1990; Rosenfeld et al., 1992; Wolfe et al., 1992). Procedures to introduce foreign genes into cells include direct transfection (Davis et al., 1986) and retroviral gene transfer (Dichek et al., 1991; Wilson et al., 1988a; Wilson et al., 1988b; Kay et al., 1992). In some cases, genetically altered cells have been reintroduced into animals (Dichek et al., 1991; Roy Chowdhury et al., 1991) where their continued function has been monitored for variable periods of time.

Recently, adenovirus-mediated gene transfer has been investigated as a means of somatic gene therapy into eukaryotic cells and into whole animals (van Doren et al., 1984a; van Doren et al., 1984b; Ghosh-Choudhury and Graham, 1987; Stratford-Perricaudet et al., 1990; Rosenfeld et al., 1991; Rosenfeld et al., 1992). A problem with adenovirus mediated gene transfer is the low level of gene product expression in target cells and a resultant lack of a functional effect.

By way of example, although adenovirus-mediated gene transfer has been used to treat ornithine transcarbamylase (OTC) deficiency in newborn mice, the expression of the ornithine transcarbamylase enzyme in the virus infected mice was typically at or below expression levels in normal mice with the result that the defect was only partially corrected (Stratford-Perricaudet et al., 1990). On the basis of those data, one would not expect that adenovirus-mediated gene transfer would be applicable to treatment of a disease requiring an overexpression of a gene product.

By way of further example, adenovirus mediated transfer of the gene for cystic fibrosis transmembrane conductance regulator (CFTR) into the pulmonary epithelium of cotton rats has been attempted, although it has not been possible to assess the biological activity of the transferred gene because there was no physiologic effect of gene transfer despite expression of the CFTR protein in lung airway cells (Rosenfeld et al., 1992). Still further, lung expression of 1-antitrypsin protein was not associated with a physiologic effect (Rosenfeld et al.,

1991). Taken together, those data do not demonstrate that adenovirus can transfer genes into cells and direct the expression of sufficient protein to achieve a physiologically relevant effect.

Targeting somatic gene therapy to particular tissues can be used in the treatment of a number of pathological conditions characterized by unregulated or pathological cellular proliferation. Somatic gene therapy can also be used to directly study the molecular mechanisms regulating abnormal or pathological cell proliferation such as occurs in metastatic cancer cells or in injured vascular smooth muscle cells.

Previous approaches to slowing down or inhibiting pathological cell proliferation have utilized protocols that ultimately result in cell death (i.e., cytotoxic procedures). The process of the present invention provides a solution to this problem by inhibiting growth without necessarily causing cell death.

Brief Summary of the Invention

The present invention provides a process of inhibiting non-neoplastic pathological cellular proliferation in a cell *in vivo*. The process comprises transducing the cell with an eukaryotic expression vector including a polynucleotide that encodes an inhibitory cell cycle regulatory protein. The vector drives expression of the polynucleotide in the cell. In a preferred embodiment, the expression vector is replication-defective adenovirus type 5. Preferably, the adenovirus lacks the early gene region E1 or the early gene regions E1 and E3.

In a particular embodiment, the cell whose proliferation is being inhibited is a vascular smooth muscle cell. In this embodiment, transducing is accomplished by infusing the expression vector into an artery that contains the vascular smooth muscle cell.

In a preferred embodiment, the inhibitory cell cycle regulatory protein is p53 or p21. In another embodiment, the inhibitory protein is a dominant-negative cell cycle regulatory protein such as a non-phosphorilatable form of Rb-1. The expression vector preferably further

includes an enhancer-promoter other than an adenovirus enhancer-promoter, where the enhancer-promoter's operatively linked to the polynucleotide that encodes the regulatory protein. In a particularly preferred embodiment, the enhancer-promoter includes the CMV promoter, an SV40 early promoter, a RSV promoter, the elongation factor promoter (EF1α), or a MCK enhancer or 4α 2 heavy chain. In another particularly preferred embodiment, the enhancer-promoter is specific for vascular smooth muscle such as an endothelin promoter or a smooth muscle -actin promoter.

In one embodiment, the present invention provides a process of inhibiting pathological proliferation of vascular smooth muscle cells. The process includes the step of increasing the level of p21 in the cells. The level of p21 is increased by increasing the expression of p21 in the cells.

In a preferred embodiment, p21 expression is increased by transforming those cells with an expression vector that contains a polynucleotide that encodes p21 operatively linked to a promoter that drives expression of p21 in the cell. A preferred expression vector is a replication-defective adenoviral vector. An especially preferred adenoviral vector is designated Adp21.

The expression of p21 in vascular smooth muscle cells can be increased in those cells either *in vivo* or *in vitro*. Where the cells are located *in vivo*, cells can be transformed with a suitable expression vector using direct *in vivo* or *ex vivo* transformation procedures.

In one embodiment, where the smooth muscle cell is located *in vivo*, the expression vector is injected into a blood vessel containing the cell. Intraarterial injection is preferred.

A process of the present invention can be used to inhibit any pathological proliferation of vascular smooth muscle cells. Preferably, the process is used to inhibit the pathological proliferation following arterial injury or during restenosis.

Thus, in another aspect, the present invention provides a process of treating a vascular proliferative disorder in an animal in need of such treatment comprising administering to the animal an effective amount of an expression vector that contains a polynucleotide that encodes p21 operatively linked to a promoter that drives expression of p21 in vascular smooth muscle.

Preferred vectors and methods of transformation are the same as set forth above.

Detailed Description of the Invention

I. The Invention

The present invention addresses one or more shortcomings in the prior art through the provision of a process for inhibiting non-neoplastic pathological cell proliferation without necessarily causing cell death. In accordance with a process of this invention, an eukaryotic expression vector construct is used to deliver a gene to the proliferating cell and thus affect expression of that gene's product. The gene product is a dominant-negative cell cycle regulatory protein. Expression of the gene product thereby alters function of those cells and inhibits the pathological proliferation. A process of the present invention can be used to inhibit proliferation of a cell situated *in vivo* in a living organism.

II. Process of Inhibiting Cell Proliferation

The present invention provides a process of inhibiting non-neoplastic pathological cellular proliferation in a cell *in vivo*, the process comprising transducing the cell with an eukaryotic expression vector comprising a polynucleotide that encodes an inhibitory cell cycle regulatory protein, the vector driving expression of the polynucleotide in the cell.

As used herein, the phrase "non-neoplastic pathological cellular proliferation" means unregulated, uncontrolled or abnormally controlled cell proliferation. That phrase is meant to incorporate all

non-neoplastic types of cellular proliferation other than the normal or physiological type of proliferation that a particular cell type undergoes. Exemplary non-neoplastic pathological cellular proliferations are fibroblast proliferation in keloid formation after surgery, prostate epithelial cell proliferation in benign prostatic hypertrophy, uterine smooth muscle and fibroblast proliferation in uterine fibroids, colonic epithelial and connective tissue cell proliferation in benign colonic polyps, benign neuromas, skin epithelial cell proliferation in hyperkeratotic skin diseases and vascular smooth muscle proliferation following vascular injury.

Exemplary vascular injuries are restenosis following balloon angioplasty of coronary arteries, restenosis following balloon angioplasty of peripheral arteries (e.g., renal, femorals, carotids), restenosis following stenting of the coronary arteries, coronary artery bypass graft restenosis and occlusion, peripheral artery bypass graft stenosis, restenosis of arterial-venous shunts in renal dialysis patients, primary pulmonary hypertension, accelerated atherosclerosis following heart transplantation and glomeruloproliferative disorders.

In a preferred embodiment, a process of the present invention is used to inhibit vascular smooth muscle proliferation during restenosis. A preferred cell, therefore, is a vascular smooth muscle cell.

The arterial wall is a complex multicellular structure that plays important roles in inflammation, coagulation, and the regulation of blood flow. Vascular smooth muscle cells are located predominantly in the arterial tunica media and are important regulators of vascular tone and blood pressure. These cells are normally maintained in a non-proliferative state *in vivo*. Arterial injury results in the migration of vascular smooth muscle cells into the intimal layer of the arterial wall where they proliferate and elaborate extracellular matrix components. This neointimal smooth muscle cell proliferative response has been implicated as important in the pathogenesis of atherosclerosis (Forrester et al., 1991; Ip et al., 1990).

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Localized arterial infection with this virus at the time of balloon angioplasty significantly reduced smooth muscle cell proliferation and neointima formation in both the rat carotid and porcine femoral artery models of restenosis. The data set forth hereinafter demonstrate an important role for Rb in regulating smooth muscle cell proliferation and a novel gene therapy approach for vascular proliferative disorders associated with arterial injury.

Although these studies clearly demonstrate the importance of Rb in controlling VSMC cell cycle progression, the molecular pathways that regulate Rb phosphorylation in VSMC remain unknown. Previous work in other cell types has suggested that the formation of cyclin D/CDK complexes with serine/threonine kinase activity is required for both Rb phosphorylation, and for the ability of cells to traverse the G1/S checkpoint of the cell cycle. Moreover, cyclin D/CDK complexes can phosphorylate Rb *in vitro*. These complexes may thus directly regulate the phosphorylation and activity of Rb in response to growth factor stimulation.

The kinase activities of the cyclin D/CDK complexes can themselves be negatively regulated by several different molecules including p16, p21, and p27. p21, also known as Cip 1 (CDK-interacting protein), Sdi1 (senescent cell-derived inhibitor), and WAF1 (wild-type p53-activated fragment), is an important inhibitor of cell cycle progression in fibroblasts and also plays a critical role in protecting cells against certain types of injury. For example, UV-induced DNA damage leads to the induction of p53 which, in turn, activates transcription of p21, resulting in cell cycle arrest and DNA repair prior to S phase-dependent chromosomal replication.

Although p21 monomers can associate with active cyclin D/CDK complexes in proliferating fibroblasts, over-expression of p21 in such fibroblasts has been shown to potently inhibit the *in vitro* kinase activity of these cyclin/CDK complexes and to arrest these cells in the G1 phase of the cell cycle. Thus, it has been suggested that p21 may inhibit cell cycle progression by inhibiting the cyclin D/CDK dependent

phosphorylation of Rb. However, to date this effect has not been demonstrated directly in mammalian cells.

In addition to its ability to inhibit the kinase activities of cyclin/CDK complexes, p21 has also been reported to bind to and inhibit the activity of the DNA polymerase α co-factor, PCNA. Recent studies have suggested that different regions of the p21 protein are required for its CDK and PCNA inhibitory activities. Thus, p21 appears to inhibit cell cycle progression by at least two independent molecular mechanisms.

As set forth hereinafter in detail, adenovirus-mediated over-expression of p21 inhibits VSMC proliferation in response to serum stimulation *in vitro*. This effect is associated with a complete inhibition of Rb phosphorylation and with the formation of p21/PCNA complexes in the cells programmed to overexpress p21. Localized infection of the arterial wall at the time of balloon angioplasty with a replication-defective adenovirus encoding p21 markedly inhibited restenosis in the rat carotid artery model of vascular injury.

Taken together, these studies demonstrate the important role of p21 in regulating VSMC proliferation *in vitro* and *in vivo*. Adenovirus-mediated overexpression of p21 thus likely represents a novel cytostatic gene therapy approach for restenosis and related vascular proliferative disorders.

Eukaryotic expression vectors are well known in the art (Sambrook et al., 1989). A preferred expression vector construct is an adenovirus vector construct. The use of adenovirus as a vector for cell transfection is well known in the art. Adenovirus vector-mediated cell transfection has been reported for various cells (Stratford-Perricaudet, et al., 1992). An adenovirus vector of the present invention is replication defective. A virus is rendered replication defective by deletion of the viral early gene region 1 (E1). An adenovirus lacking an E1 region is competent to replicate only in cells, such as human 293 cells, which express adenovirus early gene region 1 genes from their

cellular genome. Thus, such an adenovirus cannot kill cells that do not express that early gene product. In a preferred embodiment, an adenovirus vector used in the present invention is lacking both the E1 and the E3 early gene regions. Techniques for preparing replication defective adenoviruses are well known in the art (See, e.g. McGrory et al., 1988, and Guzman et al., 1982).

It is believed that any adenovirus vector can be used in the practice of the present invention. Thus, an adenovirus vector can be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material for production of a replication-defective adenovirus vector.

An adenovirus is engineered to contain a coding DNA sequence for use as a vector. Such a recombinant adenovirus has been described by Gluzman et al., 1982. Individual DNA sequences such as cDNAs that encode a gene product are inserted into the adenovirus to create a vector construct. In a preferred embodiment, therefore, a coding sequence for a gene product is introduced or incorporated into an adenovirus at the position from which the E1 coding sequences have been removed. However, the position of insertion within the adenovirus sequences is not critical to the present invention. A coding sequence can also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described previously by Karlsson et al. (1986). Preferably, the E1 region of adenovirus is replaced by the coding DNA sequence or gene.

The resulting adenovirus vector is co-transfected into 293 cells together with a plasmid carrying a complete adenovirus genome to propagate the adenovirus. An exemplary such plasmid is pJM17. Co-transfection is performed in accordance with standard procedures well known in the art. By way of example, 293 cells are cultured in Dulbecco's modified Eagle's medium containing fetal calf serum. Confluent cultures are split the day before calcium phosphate cotransfection of plasmids. After addition of the DNA to the cells, the cells are shocked (e.g., a 15% glycerol shock) to boost transfection

efficiency and the cells are overlaid with agar in DMEM containing fetal calf serum, penicillin, streptomycin sulfate, and other antibiotics or antifungal agents as needed. Monolayers are incubated until viral plaques appear (about 5-15 days).

These plaques are picked, suspended in medium containing fetal calf serum, and used to infect a new monolayer of 293 cells. When greater than 90% of the cells showed infection, viral lysates are subjected to a freeze/thaw cycle and designated as primary stocks. The presence of recombinant virus is verified by preparation of viral DNA from infected 293 cells, restriction analysis, and Southern blotting. Secondary stocks are subsequently generated by infecting 293 cells with primary virus stock at a multiplicity of infection of 0.01 and incubation until lysis.

The particular cell line used to propagate the recombinant adenoviruses of the present invention is not critical to the present invention. Recombinant adenovirus vectors can be propagated on, e.g., human 293 cells, or in other cell lines that are permissive for conditional replication-defective adenovirus infection, e.g., those which express adenovirus E1 gene products "in trans" so as to complement the defect in a conditional replication-defective vector. Further, the cells can be propagated either on plastic dishes or in suspension culture, to obtain virus stocks thereof.

A coding sequence can comprise introns and exons so long as the coding sequence comprises at least one open reading frame for transcription, translation and expression of that polypeptide. Thus, a coding sequence can comprise a gene, a split gene or a cDNA molecule. In the event that the coding sequence comprises a split gene (contains one or more introns), a cell transformed or transfected with a DNA molecule containing that split gene must have means for removing those introns and splicing together the exons in the RNA transcript from that DNA molecule if expression of that gene product is desired.

A coding sequence in an adenovirus vector can code for any dominant-negative cell cycle regulatory protein. As used herein, the phrase "dominant negative cell cycle regulatory protein" means a protein that acts to inhibit cell proliferation or arrest cell growth at any of the cell cycle stages (e.g., S, G_0, or G_1). Such dominant-negative cell cycle regulatory proteins are well known in the art. Exemplary and preferred such proteins are the p53 gene product, the p21 gene product and the retinoblastoma gene product (Rb).

The classical tumor suppressor genes, p53 and Rb have each been shown to play important roles in regulating cell cycle progression in a number of mammalian cell types (Simons et al., 1992; Morishita et al., 1993; Barr and Leiden, 1994; Hollingsworth et al., 1993; Perry and Levine, 1993; Helin and Harlow, 1993; Friend, 1994). Moreover, recent evidence suggests that p53-dependent G_1 arrest of cell cycle progression is mediated at least in part through Rb or Rb-like proteins (Slebos et al., 1994). The inactivation of Rb or p53 either by mutation or by viral oncoproteins results in unregulated proliferation and tumorigenesis in both animals and humans (Lee et al., 1988; Lee et al., 1987; Friend et al., 1987; Williams et al., 1994; Malkin et al., 1990; Livingstone et al., 1992; Donehower et al., 1992).

The studies described hereinafter show that Rb plays a critical role in regulating the proliferation of vascular smooth muscle cells both in response to growth factor stimulation *in vitro*, and to injury *in vivo* and indicate significant parallels between the molecular mechanisms underlying carcinogenesis and those leading to human vascular proliferative disorders such as atherosclerosis and restenosis. In this regard, *in vivo* gene transfer of dominant-negative cell cycle regulatory proteins into a number of different cell types are likely be useful for the treatment of a variety of human diseases associated with uncontrolled cellular proliferation.

The retinoblastoma gene product (Rb) is an important inhibitor of cell cycle progression in many mammalian cell types (Hollingsworth et al., 1993; Perry and Levine, 1993; Helin and Harlow, 1993; Friend,

1994). For example, in resting (G_0) peripheral blood T cells, Rb is unphosphorylated, and, in that state, binds to and inactivates a set of cellular transcription factors including E2F and Elf-1 that are important for cell cycle progression (Chen et al., 1989; DeCaprio et al., 1992; Kovesdi et al., 1986; Wang et al., 1993; Buchkovich et al., 1989; Mihara et al., 1989; Bandara et al., 1991). Following T cell activation, Rb becomes rapidly phosphorylated causing disruption of the Rb/E2F and Rb/Elf-1 complexes (Chen et al., 1989; DeCaprio et al., 1992; Kovesdi et al., 1986; Wang et al., 1993; Buchkovich et al., 1989; Mihara et al., 1989; Bandara et al., 1991; Huang et al., 1991; Kaeline et al., 1991). The release of these Rb-associated transcription factors is associated with progression through the G_1/S checkpoint of the cell cycle and subsequent T cell proliferation.

The importance of Rb in regulating normal cell cycle progression has been underscored by the finding that mutations and deletions of Rb are associated with abnormal cell cycle progression and malignancies in both mice and humans (Lee et al., 1988; Lee et al., 1987; Friend et al., 1987). In addition, several viral oncogenes including the SV40 large T antigen, adenovirus E1A and human papillomavirus E7 proteins bind to Rb, thereby competitively disrupting the Rb-transcription factor complexes and leading to unregulated cell proliferation (DeCaprio et al., 1988; Ewen et al., 1989; Ludlow et al., 1989; Dyson et al., 1989). The polynucleotide sequences of p21, p53 and Rb are well known in the art.

A coding sequence of an adenovirus vector construct is preferably operatively linked to an enhancer-promoter other than an adenovirus enhancer-promoter. A promoter is a region of a DNA molecule typically within about 100 nucleotide pairs in front of (upstream of) the point at which transcription begins (i.e., a transcription start site). That region typically contains several types of DNA sequence elements that are located in similar relative positions in different genes. As used herein, the term "promoter" includes what is referred to in the art as an upstream promoter region, a promoter region

or a promoter of a generalized eukaryotic RNA Polymerase II transcription unit.

Another type of discrete transcription regulatory sequence element is an enhancer. An enhancer provides specificity of time, location and expression level for a particular encoding region (e.g., gene). A major function of an enhancer is to increase the level of transcription of a coding sequence in a cell that contains one or more transcription factors that bind to that enhancer. Unlike a promoter, an enhancer can function when located at variable distances from transcription start sites so long as a promoter is present.

As used herein, the phrase "enhancer-promoter" means a composite unit that contains both enhancer and promoter elements. An enhancer-promoter is operatively linked to a coding sequence that encodes at least one gene product. As used herein, the phrase "operatively linked" means that an enhancer-promoter is connected to a coding sequence in such a way that the transcription of that coding sequence is controlled and regulated by that enhancer-promoter. Means for operatively linking an enhancer-promoter to a coding sequence are well known in the art. As is also well known in the art, the precise orientation and location relative to a coding sequence whose transcription is controlled, is dependent *inter alia* upon the specific nature of the enhancer-promoter. Thus, a TATA box minimal promoter is typically located from about 25 to about 30 base pairs upstream of a transcription initiation site and an upstream promoter element is typically located from about 100 to about 200 base pairs upstream of a transcription initiation site. In contrast, an enhancer can be located downstream from the initiation site and can be at a considerable distance from that site.

An enhancer-promoter used in a vector construct of the present invention can be any enhancer-promoter that drives expression in a target cell. In an example set forth hereinafter, the human cytomegalovirus (CMV) immediate early gene promoter has been used to result in high-level expression of a gene. However, the use of other

viral or mammalian cellular promoters which are well-known in the art is also suitable to achieve expression of the gene product provided that the levels of expression are sufficient to achieve a physiologic effect. Exemplary and preferred enhancer-promoters are the CMV promoter, the Rous sarcoma virus (RSV) promoter, the EF1α promoter, the muscle-specific creatine kinase (MCK) enhancer or the 4F2 heavy chain enhancer (Zambetti et al., 1992; Yi et al., 1991 and Sternberg et al., 1988).

By employing an enhancer-promoter with well-known properties, the level and pattern of gene product expression can be optimized. For example, selection of an enhancer-promoter that is active specifically in vascular smooth muscle permits tissue-specific expression of the gene product. Preferably a vascular smooth muscle specific enhancer-promoter is an endothelin promoter (See e.g., Lee et al., 1990 and Bloch et al., 1989) or a smooth muscle -actin promoter (See e.g., Foster et al., 1992 and Blank et al., 1992). Still further, selection of an enhancer-promoter that is regulated in response to a specific physiologic signal can permit inducible gene product expression.

A coding sequence of an adenovirus vector construct is operatively linked to a transcription terminating region. RNA polymerase transcribes an encoding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA).

Transcription-terminating regions are well known in the art. A preferred transcription-terminating region used in an adenovirus vector construct of the present invention preferably comprises a polyadenylation signal of SV40 bovine growth hormone 3 or the protamine gene.

Transducing is accomplished by delivering the expression vector to the cell or cells whose pathological proliferation is to be inhibited. Delivering is accomplished by infusing the vector into an artery that perfuses the target cell. In this way, delivery is localized to the target tissues. Targeted delivery of adenovirus vector containing a coding sequence for ß-galactosidase (ß-gal) to cardiac muscle cells was accomplished by infusing that construct via a catheter placed into the left coronary artery or coronary sinus ostium (See Examples 1, 2, and 5 hereinafter). Targeted delivery of adenovirus vector containing a coding sequence for Rb or p21to vascular smooth muscle cells was accomplished by infusing that construct via a catheter placed into an artery (See Examples 4 and 5 hereinafter).

It should also be pointed out that because the adenovirus vector employed in replication defective, it is not capable of replicating in the cells that are ultimately infected. Moreover, it has been found that the genomic integration frequency of adenovirus is usually fairly low. Thus, where continued treatment is required it may be necessary to reintroduce the virus every 6 months to a year. In these circumstances, it may therefore be necessary to conduct long term therapy, where expression levels are monitored at selected intervals.

An adenovirus vector construct is typically delivered in the form of a pharmacological composition that comprises a physiologically acceptable carrier and the adenovirus vector construct. An effective expression-inducing amount of an adenovirus vector construct is delivered. As used herein, the term "effective expression-inducing amount" means that number of virus vector particles necessary to effectuate expression of a gene product encoded by a coding sequence contained in that vector. Means for determining an effective expression-inducing amount of an adenovirus vector construct are well known in the art. An effective expression-inducing amount is typically from about 10^7 plaque forming units (pfu) to about 10^{15} pfu, preferably from about 10^8 pfu to about 10^{14} pfu and, more preferably, from about 10^9 to about 10^{12} pfu.

As is well known in the art, a specific dose level for any particular subject depends upon a variety of factors including the infectivity of the adenovirus vector, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, and the severity of the particular disease undergoing therapy.

In that adenovirus is a virus that infects humans, there can be certain individuals that have developed antibodies to certain adenovirus proteins. In these circumstances, it is possible that such individuals might develop an immunological reaction to the virus. Thus, where an immunological reaction is believed to be a possibility, one can first test the subject to determine the existence of antibodies. Such a test can be performed in a variety of accepted manners, for example, through a simple skin test or through a test of the circulating blood levels of adenovirus-neutralizing antibodies. In fact, under such circumstances, one may desire to introduce a test dose of on the order of $x\ 10^6$ to 1×10^6 or so virus particles. Then, if no untoward reaction is seen, the dose is elevated over a period of time until the desired dosage is reached, such as through the administration of incremental dosages of approximately an order of magnitude.

The following examples illustrate particular embodiments of the present invention and are not limiting of the specification and claims in any way.

EXAMPLE 1: Making Of Adenovirus Vector Construct

This example describes the use of recombinant replication defective adenoviruses in the preparation of virus vector constructs comprising a coding DNA sequence.

A. Beta-galactosidase

Recombinant adenovirus (Guzman et al., 1982) containing distinct cDNAs (AdCMV-cDNA) were prepared in accordance with standard techniques well known in the art. E.coli ß-galactosidase cDNA carrying the SV40 T antigen nuclear targeting signal (Bonnerot

et al., 1987) was inserted into pAdCMV to create a distinct construct comprising the cytomegalovirus (CMV) promoter, the ß-Gal cDNA and a polyadenylation signal from either the SV40 virus or the mouse protamine gene, and flanked by adenovirus type 5 sequences. In this construct, the E1 and E3 region of adenovirus were deleted and the E1 region was replaced by the ß-Gal encoding sequence.

The resulting plasmid, designated AdCMVß-gal, was cotransfected into 293 cells. Co-transfection was performed as follows: 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum. Confluent dishes were split to non-confluent flasks the day before cotransfection with pAdCMVß-gal. Monolayers were incubated until the appearance of viral plaques.

These plaques were picked, suspended in DMEM containing 2% fetal calf serum and used to infect a new monolayer of 293 cells. When greater than 90% of the cells showed infection, viral lysates were subjected to a freeze/thaw cycle and were designated as primary stocks. Recombinant virus with the correct structure was verified by preparation of viral DNA from productively-infected 293 cells, restriction analysis, and Southern blotting. Secondary stocks were subsequently generated by infecting 293 cells with primary virus stock and incubation until lysis.

The large scale production of recombinant adenovirus was performed in 293 cells. Infected cells were lysed 48 hours post-infection. Virus-containing extracts were centrifuged to remove debris before precipitation of the virus. Virus was collected by centrifugation, resuspended in isotonic medium, purified, and sterilized.

Alternatively, precipitated virus can be resuspended in 50mM Tris-HCl pH 7.8 containing CsCl (d=1.10 g/ml), layered over a step-gradient formed of 2 ml CsCl (d=1.40) and 3 ml of CsCl (d=1.30), and centrifuged 2 hours at 20,000 rpm at 10%C in a Sorvall TH641 rotor. Virus is collected from the lower interface and dialyzed overnight at 4%C versus isotonic saline.

B. Retinoblastoma gene product

Adenovirus vectors were constructed by recombination between plasmid DNA and XbaI/ClaI digested Ad5Sub360 adenovirus DNA in 293 cells as described previously (Barr et al., 1994; Berkner, 1988). Recombinants were plaque purified three times to avoid contamination with replication-competent virus. High titer adenovirus stocks were prepared by infecting 293 cells with 2-5 pfu/cell of virus. Viral lysates were purified by centrifugation in discontinuous CsCl gradients (Barr et al., 1994). Viral titers were determined from the A_{260} of the purified virus preparation (1 OD= 10^{10} pfu/ml). Primary cultures of rat aortic vascular smooth muscle cells were isolated and grown.

Passage 3 vascular smooth muscle cells were placed in serum free medium for 48 hrs. and then infected with 5 pfu/cell of AdβAc.lacZ or AdEF1HAΔRb for 1 hr. 24 hrs. post-infection, the cells were stimulated to proliferate by exposure to 10% Fetal Calf Serum (FCS). Western blots were performed using 50 mg of cell lysate per lane (Chen et al., 1989; DeCaprio et al., 1992; Kovesdi et al., 1986; Wang et al., 1993; Buchkovich et al., 1989; Mihara et al., 1989; Bandara et al., 1991) and a mouse -human Rb mAb (Pharmingen) (1:2000 dilution) or a mouse -HA mAb (Boehringer Mannheim, Indianapolis, IN) (1:2000 dilution). For ^{3}H-thymidine assays, cultured vascular smooth muscle cells were pulse-labeled for 4 hrs. with medium containing 1 mCi/ml methyl-^{3}H thymidine (5 Ci/mmol, 1 mCi/ml, Amersham) (Owens et al., 1986). ^{3}H-thymidine incorporation was determined using a Packard Model 1900 TR Liquid Scintillation Spectrophotometer.

EXAMPLE 2: Functional Expression of AdCMVβ-gal

Adult rabbits were anesthetized and a catheter was inserted into the right carotid artery or internal jugular vein. The tip of the catheter was advanced under fluoroscopic guidance to the left coronary artery or coronary sinus ostium.

About 1000 µg to 1500 µg of adenovirus vector construct pAdCMVß-gal, prepared in accordance with the procedures of Example 1, were suspended in physiologically buffered saline. About 2×10^9 plaque forming units (pfu) of pAdCMVß-gal were infused into the indwelling catheter. The catheter was removed, all incisions were closed and the rabbits allowed to recover. Rabbits were killed 5 to about 21 days after injection. The heart and associated vasculature was removed and examined histochemically for ß-gal activity as set forth below.

Three-millimeter cross sections of the left ventricle were fixed for 5 minutes at room temperature with 1.25% glutaraldehyde in PBS, washed three times at room temperature in PBS, and stained for ß-galactosidase activity with X-gal (Biorad) for 4-16 hours as described by Nabel et al. (Nabel, et al., (1989). The 3-mm sections were embedded with glycomethocyrlate, and 4-7 &m sections were cut and counterstained with hematoxylin and eosin as described previously. (Nabel, et al., 1989). Photomicroscopy was performed using Kodak Ektachrome 200 file and Leitz Laborlux D and Wild M8 microscopes. ß-Gal activity was observed in coronary vascular smooth muscle and in cardiac muscle cells.

EXAMPLE 3: Regulation of Rb Expression in Vascular Smooth Muscle

To study the expression and regulation of Rb in vascular smooth muscle cells, cultured primary rat aortic smooth muscle cells were arrested in the G_0/G_1 phase of the cell cycle by incubation in serum-free medium for 96 hours, and then stimulated to proliferate by exposure to 10% FCS. The expression and phosphorylation of Rb was assessed by Western blot analysis of whole cell extracts. Primary rat aortic smooth muscle cells were isolated and grown as described previously (Blank et al., 1988). Passage 3 vascular smooth muscle cells were placed in serum free medium (50% DMEM, 50% Ham's F-12, 292 mg/ml l-glutamine, 5 mg/ml insulin, 5 mg/ml transferrin, 5 ng/ml selenious acid) for 96 hours and then stimulated by incubation in 45%

DMEM, 45% Ham's F-12, 10% FCS. Cell lysates were prepared as described previously (Chen et al., 1989; DeCaprio et al., 1992; Kovesdi et al., 1986; Wang et al., 1993; Buchkovich et al., 1989; Mihara et al., 1989; Bandara et al., 1991) and protein corresponding to 6 X 10^5 cells from each sample was subjected to Western blot analysis with an -Rb mAb (Pharmingen, San Diego, CA) (1:2000 dilution). A peroxidase-labeled, goat -mouse IgG antiserum (1:2000 dilution) (Gibco BRL) was used for detection in conjunction with the ECL chemiluminescence system (Amersham, Northbrook, IL).

Following serum withdrawal, more than 85% of the cultured vascular smooth muscle cells were arrested in G_0 or G_1 of the cell cycle as assayed by propidium iodide staining and FACS analysis. These quiescent cells contained exclusively unphosphorylated Rb. Serum stimulation of these cells was associated with their progression into the S phase of the cell cycle, and the concomitant, progressive phosphorylation of Rb during the first 24 hours after stimulation. This pattern of Rb phosphorylation was consistent with the hypothesis that Rb plays an important role in regulating vascular smooth muscle cell proliferation in response to growth factor stimulation.

To directly test the role of Rb in regulating cell cycle progression in smooth muscle cells, we constructed a replication-defective adenovirus vector, AdEF1HAΔRb, that encodes a non-phosphorylatable, constitutively active form of human Rb (hRb) containing a 10 amino acid N-terminal epitope tag from the influenza hemagglutinin molecule (HA). In this vector, transcription of Rb is controlled by the cellular EF1-a promoter and the 4F2HC enhancer. This mutant form of Rb (HAΔRb) has been reported previously to inhibit E2F- and Elf-1-dependent transcription in P19 (Hamel et al., 1992) and T cells (Chen et al., 1989; DeCaprio et al., 1992; Kovesdi et al., 1986; Wang et al., 1993; Buchkovich et al., 1989; Mihara et al., 1989; Bandara et al., 1991), respectively. Replication-defective adenovirus was found to transduce greater than 90% of cultured primary rat aortic vascular smooth muscle cells *in vitro*. To demonstrate expression of

HAΔRb following adenovirus-mediated gene transfer, cultured rat aortic smooth muscle cells were infected with 20 plaque forming units (pfu)/cell of AdEF1HAΔRb and whole cell extracts were subjected to Western blot analysis with both -hRb and -hemagglutinin epitope (-HA) antibodies. Control cultures were infected with 20 pfu/cell of AdβAc.lacZ, a replication-defective adenovirus containing the bacterial lacZ gene under the control of the chicken β-actin promoter and cytomegalovirus enhancer (Kozarsky et al., 1993). Infection with AdEF1HAΔRb, but not with AdβAc.lacZ, resulted in expression of a 107 kD protein that was reactive with both the -HA and -hRB antibodies. This protein co-migrated with *in vitro* translated HAΔRb run in parallel on the same Western blot. The lack of detectable Rb in the AdβAc.lacZ-infected vascular smooth muscle cells reflects the fact that the -hRb antibody used in these experiments recognizes human Rb more efficiently than rat Rb and that AdEF1HAΔRb infection results in high levels of expression of human Rb.

To determine the effect of overexpression of HAΔRb on growth factor-stimulated smooth muscle cell proliferation *in vitro*, quiescent rat aortic smooth muscle cells were infected with 20 pfu/cell of AdEF1HAΔRb and then stimulated to proliferate by incubation in 10% FCS. Control cultures were infected with AdβAc.lacZ or were left uninfected prior to serum stimulation. Serum stimulation caused the rapid proliferation of the uninfected or AdβAc.lacZ-infected vascular smooth muscle cells. During the first 48 hours after stimulation, these cells underwent approximately 3 doublings. In contrast, infection with AdEF1HAΔRb caused a greater than 90% reduction in smooth muscle cell proliferation. Of note, both the AdβAc.lacZ and AdEF1HAΔRb-infected cells were more than 97% viable at the end of the experiment as determined by trypan blue exclusion. Thus, the lack of proliferation seen following AdEF1HAΔRb infection represented cell cycle arrest as opposed to cell killing in these experiments.

Rb has been shown to block cell cycle progression at the G_1/S transition (Goodrich et al., 1991). To determine whether the

AdEFHAΔRb-infected vascular smooth muscle cells were arrested prior to the S phase of the cell cycle, ^{3}Hthymidine incorporation was measured in these cells following serum stimulation. Serum stimulation of both the uninfected and AdßAc.lacZ-infected vascular smooth muscle cells was associated with significant increases in ^{3}H-thymidine incorporation during the first 24 to 48 hours after stimulation. In contrast, infection with AdEF1HAΔRb inhibited ^{3}H-thymidine incorporation by more than 90% in these cells. This finding was consistent with the hypothesis that overexpression of HAΔRb arrests smooth muscle cell proliferation prior to entry into the S phase of the cell cycle.

EXAMPLE 4: Regulation of Rb Expression in Restenosis

Two established animal models of restenosis were used in these experiments. The rat carotid artery injury model represents a well characterized, reproducible vascular proliferative disorder that is dependent on smooth muscle cell migration and proliferation (Simons et al., 1992; Morishita et al., 1993; Barr and Leiden, 1994; Clowes and Reidy, 1983). Balloon angioplasty of the porcine femoral artery produces a neointimal lesion that has been used as a model of human vascular proliferative disease. Importantly, both the size and structural organization of this vessel closely resemble those of the human coronary arteries (Prescott et al., 1991; Reitman et al., 1982; Weiner et al., 1985; Ohno et al., 1994).

Two experiments were performed to assess the efficiency of *in vivo* gene transfer into vascular smooth muscle cells following adenovirus infection. First, rat carotid arteries were injured by balloon angioplasty and then infected for 5 minutes with 2 X10^{9} pfu of AdßAc.nlacZ (which is similar to AdßAc.lacZ but encodes a nuclear localization signal along with the bacterial lacZ) (Kozarksy et al., 1993). Five days after infection, arteries were stained with X-gal to detect nuclear-localized ß-galactosidase activity. AdßbAc.nlacZ infection resulted in efficient gene transfer into vascular smooth muscle cells at

the site of balloon injury. No nuclear blue staining was observed in control arteries infected with AdBglII, a replication-defective adenovirus that does not encode a recombinant gene, or in uninfected balloon injured arteries.

These results were consistent with previous reports that have demonstrated efficient *in vivo* gene transfer into rat carotid, rabbit coronary, and porcine femoral arterial vascular smooth muscle cells following infection with replication-defective adenoviruses (Prescott et al., 1991; Reitman et al., 1982; Weiner et al., 1985; Ohno et al., 1994; Barr et al., 1994; Guzman et al., 1993).

To demonstrate *in vivo* gene transfer of HAΔRb following AdEF1HAΔRb infection, rat carotid arteries were injured by balloon angioplasty and then infected for 5 minutes with 2 X 10^9 pfu of AdEF1HAΔRb. Five days after infection, carotid arteries were assayed for HAΔRb RNA by reverse transcriptase PCR. RNA was prepared as described previously using the acid guanidinium-phenol method (Chomczynski, 1993). To ensure that the RNA samples were free of DNA contamination, all extracted samples were subjected to digestion with 10 U DNase I for 30 min. at 37°C. First strand cDNA synthesis was performed using 1 mg of RNA and a commercially available kit (Perkin Elmer, Norwalk, CT) in the presence and absence of reverse transcriptase (Karpinski et al., 1992). PCR was performed as described previously (Barr et al., 1994) using primers specific for the HAΔRb cDNA [AAGCTTCCCGGGGAATTCACCATGGGGTACCCATACGATGTTCCAG ATTACG (sense)(SEQ ID NO:1) and ATAGCATTATCAACCTTGGTACTGG (antisense)(SEQ ID NO:2)] or the mouse ß-actin cDNA [GTGACGAGGCCCAGAGCAAGAG (sense)(SEQ ID NO:3) and AGGGGCCGGACTCATCGTACTC (antisense)(SEQ ID NO:4)]. Southern blot analysis was performed using a radiolabeled probe corresponding to bp 1 to 392 of the HAΔRb cDNA (Barr et al., 1994).

HAΔRb RNA was detected in the AdEF1HAΔRb-infected but not in the control AdBglII-infected or contralateral uninfected carotid arteries. To determine the effects of HAΔRb expression on restenosis, rat carotid arteries were subjected to balloon angioplasty and immediately infected with either 2 X 10^9 pfu of AdEF1HAΔRb or a control AdBglII virus. A third set of arteries was treated with vehicle (HEPES-buffered saline) alone. Two assays were used to measure smooth muscle cell proliferation *in vivo*. First, to directly determine the numbers of proliferating medial smooth muscle cells, AdEF1HAΔRb-infected and control arteries were stained for 5'-bromodeoxyuridine (BrdU) (Simons et al., 1992; Morishita et al., 1993; Barr and Leiden, 1994; Lindner et al., 1992) incorporation 3 days after balloon injury. Carotid arteries of adult rats were injured by balloon angioplasty. Immediately following balloon injury, arteries were infected with 2 X 10^9 pfu of either AdBglII or AdEF1HAΔRb, or were left uninfected. Animals received subcutaneous injections of 25 mg/kg of 5'-bromodeoxyuridine at 12 hour intervals starting 24 hours following injury for a total of 4 doses. Carotid arteries were fixed in situ by intravascular administration of 4% paraformaldehyde, paraffin-embedded, and sectioned. Deparaffinized 5 mm sections were treated with 3% H_2O_2 in methanol and permeabilized by incubation in 0.4% pepsin and 3.3 M HCl. Treated sections were blocked in 1.5% horse serum, incubated with a 1:100 dilution of an -BrdU mAb (Becton-Dickinson, San Jose, CA). The sections were then incubated with a 1:200 dilution of biotinylated horse -mouse Ig antiserum, followed by avidin-conjugated horseradish peroxidase (Vectastain Elite ABC kit, Vector laboratories, Burlingame, CA). Sections were then treated with diaminobenzidine (DAB) and counterstained with Hematoxylin and Eosin. Statistical analyses were performed using Sigmaplot (Jandel Scientific, Corte Madera, CA).

Control experiments using balloon-injured uninfected arteries demonstrated that medial smooth muscle cell proliferation reached a peak within 4 days after injury, with as many as 40% of the medial vascular smooth muscle cells labeling with BrdU. Neointimal smooth

muscle cell proliferation demonstrated a slightly delayed time course with approximately 90% of the neointimal vascular smooth muscle cells staining with BrdU 6 days after balloon injury. Approximately 45% of the medial vascular smooth muscle cells in the vehicle-treated and AdBglII-infected arteries were labeled with BrdU 3 days after balloon injury. In contrast, infection with AdEF1HAΔRb resulted in a 67% reduction in BrdU-staining medial cells. These data show that localized arterial infection with AdEF1HAΔRb at the time of balloon angioplasty efficiently inhibited the proliferation of medial vascular smooth muscle cells before these cells had migrated into the neointima.

In a second series of experiments, restenosis, as determined by the neointima to media area ratio (I/M) (Simons et al., 1992; Morishita et al., 1993; Barr and Leiden, 1994; Clowes and Reidy, 1983; Prescott et al., 1991; Reitman et al., 1982; Weiner et al., 1985; Ohno et al., 1994; Lindner et al., 1992), was measured 20 days after balloon injury in the rat carotid model. Rat carotid arteries were injured with a balloon catheter and treated with vehicle alone, or infected with 2 X 10^9 pfu of AdBglII or AdEF1HAΔRb. Twenty days following injury, carotid arteries were harvested and tissue sections were stained with Hematoxylin and Eosin. Neointimal and medial boundaries were determined by digital planimetry of tissue sections using the MOCHA program (Jandel Scientific, Corte Madera, CA) on a Gateway 486 computer. The neointimal and medial cross-sectional areas were measured from six sections of each artery and the mean of these six determinations was used to calculate the neointimal to medial cross-sectional ratio for each animal.

Uninfected and control AdBglII-infected arteries had I/M area ratios of 1.4 0.1 and 1.2 0.1, respectively. In contrast, the AdEF1HAΔRb-infected arteries demonstrated a 42% reduction in the I/M ratio as compared to the AdBglII-infected controls and a 50% reduction as compared to uninfected control arteries. Thus, overexpression of HAΔRb following adenovirus-mediated *in vivo* gene

transfer at the time of injury resulted in significant reductions in both vascular smooth muscle cell proliferation and restenosis in the rat carotid artery model of balloon angioplasty. To determine the effects of AdEF1HAΔRb infection on the re-endothelialization of the carotid arteries following balloon angioplasty, arteries harvested 20 days after injury were stained with an -von Willebrand Factor mAb (Hruban et al., 1987). Rat carotid arteries were harvested 21 days after balloon injury and infection with AdEF1HAΔRb and embedded in paraffin. 5 mm sections were stained with a commercially available -vWF mAb (Dako, Santa Barbara, CA) (Hruban et al., 1987) followed by a biotinylated goat anti-mouse IgG using an automatic immunostainer (Ventana Immunosystems, Tucson, AZ). Slides were developed with avidin-conjugated alkaline phosphatase and fast red-naphthol (Ventana Immunosystems) according to the manufacturer's instructions.

These experiments demonstrated efficient reendothelialization of the injured arterial segment. This lack of inhibition of reendothelialization following AdEF1HAΔRb infection may reflect the transient nature of adenovirus-mediated recombinant gene expression *in vivo* (Barr et al., 1994) and/or the repopulation of the injured vessel segment by endothelial cells that have migrated from arterial sites located outside of the adenovirus-infected segment.

To demonstrate that the reduction in restenosis seen in the rat carotid artery model was not species or model-dependent, we also tested the effects of AdEF1HAΔRb infection in the porcine femoral artery model of restenosis. In these experiments a double balloon catheter was used to infuse 7 X 10^9 pfu of either AdBglII or AdEF1HAΔRb into a localized segment of the femoral artery immediately following balloon angioplasty. Twenty-one days after balloon angioplasty, the infected arteries were harvested and neointima to media area ratios were determined. AdBglII-infected arteries had I/M ratios of 0.68 0.05. The AdEF1HAΔRb-infected arteries demonstrated a 47% reduction in the I/M area ratio as compared to the AdBglII-infected controls. Thus, AdEF1HAΔRb

infection significantly reduces neointimal formation in two different animal models of restenosis.

Previous studies have demonstrated inflammatory responses and clinical toxicity associated with the *in vivo* administration of replication-defective adenovirus vectors (Simon et al., 1993). AdEF1HAΔRb infection did not result in increased vascular inflammation or cell necrosis as compared to vehicle-treated or AdBglII-treated control arteries. Moreover, routine serum chemistries including electrolytes, liver function tests, complete blood counts, and clotting parameters were all normal in rats 21 days following intra-arterial infusions of AdEF1HAΔRb. Pigs receiving AdEF1HAΔRb demonstrated a mild reduction in serum phosphate as compared to saline control-treated animals. The mechanism of this reduction remains unclear. Autopsies of the AdEF1HAΔRb treated rats and pigs failed to reveal significant organ inflammation or pathology. Thus, with the exception of mild hypophosphatemia, localized vascular infection with AdEF1HAΔRb did not result in significant toxicity in two mammalian species.

The data demonstrate that adenovirus-mediated gene transfer of a constitutively active form of Rb is sufficient to significantly inhibit vascular smooth muscle cell proliferation and neointimal formation in two animal models of restenosis. Previous reports have suggested that antisense oligonucleotides directed at c-myb or PCNA + cyclin A may also be effective inhibitors of restenosis *in vivo* (Simons et al., 1992; Morishita et al., 1993; Barr and Leiden, 1994; Clowes and Reidy, 1983; Lindner et al., 1992). In addition, two recent reports have demonstrated a reduction in restenosis following *in vivo* gene transfer of the Herpes Simplex Virus thymidine kinase (HSV TK) gene to the arterial wall followed by the systemic administration of ganciclovir (Prescott et al., 1991; Reitman et al., 1982; Weiner et al., 1985; Ohno et al., 1994). In contrast to previous studies using non-specific cytotoxic approaches such as HSV TK gene transfer, these data provide novel information concerning the molecular mechanisms that regulate vascular smooth muscle cell proliferation *in vitro* and *in vivo*. Moreover, unlike cytotoxic

protocols such as those involving the HSV TK gene, the presently described cytostatic therapy using Rb gene transfer has the advantage of arresting cell cycle progression without causing cell necrosis or inflammation in the vessel wall. Finally, as opposed to previously reported anti-sense oligonucleotide approaches, adenovirus-mediated gene transfer can be performed using percutaneous catheter-based techniques. The data further demonstrate the safety, efficacy and feasibility of this approach in two different animal models of restenosis.

EXAMPLE 5: Expression of p21

A. Construction and Purification of Recombinant Adenoviruses.

Adp21 and AdlacZ are E1- and E3-deleted replication-defective adenovirus vectors derived from Ad5 sub360. Adp21 encodes the human p21 cDNA under the transcriptional control of the human elongation factor-1α (EF1α) gene promoter and the human 4F2 heavy chain gene transcriptional enhancer. AdlacZ contains the bacterial lacZ gene under the transcriptional control of the chicken ß-actin gene promoter and the cytomegalovirus transcriptional enhancer. Both viruses were prepared and grown as high titer stocks in 293 cells. All virus stocks were purified by centrifugation in discontinuous CsCl gradients and dialyzed against a Hepes-buffered saline solution.

B. Cell Culture and Infection

Isolation and infection of primary rat aortic VSMCs were performed as described in Example 1. For measurements of cell proliferation, DNA synthesis, and cell cycle analysis passage 3, VSMCs were incubated in serum-free medium for 48 hours prior to infection with either Adp21 or AdlacZ. This protocol resulted in 70-90% of the cultured cells accumulating in G0 + G1 of the cell cycle as assessed by propidium iodide staining and FACS analysis.

Twenty four hours after infection, Adp21- or AdlacZ-infected cells were stimulated to proliferate by incubation in growth medium (45%

DMEM, 45% Hams F-12, and 10% FBS) (GibcoBRL, Grand Island, NY). To assay ^{3}H-thymidine incorporation, VSMCs were pulse-labeled for 4 hours in growth medium containing methyl-3H thymidine (1uCi/ml, 2 Ci/mmol; Amersham, Arlington Heights, IL). Each experiment was performed in triplicate in 6-well tissue culture plates (Falcon, Franklin Lakes, NJ).

Adp21-infected VSMCs expressed markedly elevated levels of p21 as compared to both uninfected and AdlacZ-infected control cells. High levels of p21 expression were observed within 24 hours of infection and the levels of p21 expression increased further between 24 and 48 hours after infection. Of note, in some experiments, both uninfected and AdlacZ-infected cell lysates displayed low levels of expression of a 21kD protein that co-migrated with *in vitro* translated human p21.

In addition, all of the cell lysates contained a faint non-specific band of slower mobility that cross-reacted with the polyclonal -p21 antiserum. It can be seen from these data that Adp21 can be used to program high-level p21 expression following infection of quiescent VSMCs *in vitro*.

The AdlacZ-infected cells proliferated rapidly during the first 48 hours following serum stimulation, undergoing approximately two doublings. In contrast, infection with Adp21 resulted in dose-dependent reductions in VSMC proliferation. At both the 24 and 48 hour time points, each of the Adp21-infected cell cultures (10, 20 and 40 PFU per cell) demonstrated significantly less proliferation than the control AdlacZ-infected cells ($P<0.02$).

Infection with 40 PFU per cell of Adp21 resulted in a 60% reduction in VSMC proliferation during the 48 hours after growth factor stimulation ($P < 0.0001$). Importantly, >97% of both the AdlacZ- and Adp21-infected cells were viable at the end of these experiments as determined by trypan blue exclusion. Thus, the inhibition of cell

proliferation observed following Adp21 infection reflected a cytostatic as opposed to a cytotoxic effect.

Infection with Adp21 resulted in statistically significant ($P < 0.05$), dose-dependent reductions in ^{3}H-thymidine incorporation which paralleled the decreased proliferative activity of these cells. Infection with 40 PFU/cell of Adp21 led to a greater than 70% reduction in growth factor-stimulated ^{3}H-thymidine incorporation as compared to the AdlacZ-infected control cells at 48 hours ($P < 0.001$) (Fig 2B).

C. Propidium Iodide Staining and FACS analysis

VSMCs were fixed overnight at 4%C with 75% ethanol and stained for 30 minutes at room temperature with propidium iodide (50 mg/ml). Cells were analyzed with a Becton Dickinson FACScan and CellFit software. A total of 4 X 10^4 cells were counted for each sample.

In a second set of experiments, quiescent VSMCs were infected with 40 PFU/cell of Adp21 or AdlacZ, and stimulated to proliferate by incubation in 10% FCS. Cell cycle progression was assayed by propidium iodide staining and FACS analysis. Prior to serum stimulation (0 hrs) 72% of the AdlacZ- and 71% of the Adp21-infected VSMCs were in the G0/G1 phases of the cell cycle. After 24 hours of serum stimulation, 60% of the AdlacZ-infected cells had progressed into the G2/M + S phases of the cell cycle (i.e., only 40% remained in G0/G1). In contrast, 69% of the Adp21 infected cells remained in G0/G1. Thus, Adp21 infection resulted in a 15-fold reduction in cell cycle progression during the first 24 hours of serum stimulation.

Similar results were observed 48 hours after serum stimulation. However, by this time point, the AdlacZ -infected cells were no longer synchrously dividing and many cells had traversed an entire cell cycle making precise quantitation of these samples difficult. Taken together, these results demonstrated that over-expression of p21 arrests VSMCs in G1, prior to entry into the S phase of the cell cycle.

To test the effects of over-expression of p21 on Rb phosphorylation in response to growth factor stimulation, quiescent (G0 + G1) primary rat VSMCs were infected with 40 PFU per cell of either Adp21 or AdlacZ, and then stimulated to synchronously enter and traverse the cell cycle by exposure to 10% FCS. The expression and phosphorylation of Rb following serum-stimulation was assessed by immunoblot analysis of whole cell extracts. Quiescent VSMCs contain only unphosphorylated Rb. Serum stimulation, which results in the rapid progression of VSMCs into the S phase of the cell cycle, was associated with the concomitant, progressive phosphorylation of Rb both in uninfected VSMC and in VSMCs infected with AdlacZ.

This finding was consistent with previous observations that have demonstrated an important role for Rb in regulating VSMC proliferation in response to growth factor stimulation. In contrast, infection of the quiescent VSMCs with Adp21 completely inhibited the growth factor-stimulated phosphorylation of Rb. Even 48 hrs after serum stimulation, the preponderance of Rb in these cells remained in the unphosphorylated state. These results demonstrated directly that over-expression of p21 results in decreased phosphorylation of Rb *in vivo*. When taken together with findings that unphosphorylated Rb inhibits VSMC cell cycle progression at the G1/S checkpoint of the cell cycle, they suggested that the ability of p21 to inhibit VSMC proliferation in response to growth factor stimulation reflects, at least in part, its ability to inhibit the cyclin/CDK-mediated phosphorylation of Rb.

In addition to its ability to inhibit the kinase activities of cyclin D/CDK complexes, p21 can bind to and inhibit the activity of the DNA polymerase cofactor, PCNA. Inhibition of PCNA-dependent DNA synthesis may therefore reflect a second mechanism by which p21 inhibits cell cycle progression. Recent studies have demonstrated that different regions of the p21 protein are involved in binding to CDKs and PCNA thereby suggesting that p21 may inhibit cell cycle progression by at least two distinct molecular mechanisms.

To test directly whether overexpression of p21 in VSMCs results in the formation of p21/PCNA complexes, quiescent rat aortic VSMCs were infected with 40 PFU per cell of Adp21 or AdlacZ and then stimulated to proliferate for 24 hours by incubation in medium containing 10% FCS. Lysates prepared from these infected cells were immunoprecipitated with an -p21 antibody and the immunoprecipitates subjected to immunoblot analyses with either -p21 or -PCNA antibodies.

Consistent with previous experiments, p21 was immunoprecipitated from the Adp21-infected but not from the AdlacZ-infected VSMCs. More importantly, PCNA was co-immunoprecipitated with p21 from Adp21-infected but not from AdlacZ-infected VSMC lysates. Thus, p21 forms complexes with PCNA following Adp21 infection of VSMCs.

D. Balloon Angioplasty and Adenovirus Infection of Rat Carotid Arteries

Adult male Sprague-Dawley rats were housed and cared for according to NIH guidelines in the Carlson animal facility of the University of Chicago. Briefly, Sprague-Dawley rats were subjected to balloon angioplasty of the left common carotid artery using a 2 French Fogarty catheter. Immediately following injury, 2 X 10^9 PFU of Adp21 or AdlacZ in a total volume of 0.2 ml was instilled into a 1 cm segment of the distal common carotid artery for 5 minutes using a 24 gauge intravenous catheter.

The rat carotid artery balloon injury model represents a well-characterized, highly reproducible vascular proliferative disorder that is dependent on VSMC proliferation and migration. Previous studies using this model have demonstrated that medial VSMC proliferation begins within 2 days of arterial injury, reaching a peak within 4 days. By 20 days after balloon angioplasty, nearly all of the injured arteries develop a stable neointimal lesion and demonstrate no evidence of

VSMC proliferation in either the neointima or the media of the vessel wall.

To determine directly the effects of p21 over-expression on neointima formation, rat carotid arteries were subjected to balloon angioplasty and immediately infected with 2 X 10^9 PFU of either Adp21 or AdlacZ. This protocol results in the transduction of more than 70% of the medial VSMC in the injured arterial segment. To demonstrate over-expression of recombinant p21 in these experiments, injured arterial segments were harvested 4 days after infection and crude lysates prepared from these arteries were assayed for p21 expression by immunoblot analysis.

E. Immunoprecipitation and Immunoblot Analyses

For immunoblot analysis of p21 expression in cultured cells, cell lysates were prepared and 100 µg total protein from each sample was fractionated by SDS-PAGE in 15% gels and subjected to immunoblot analysis using a rabbit polyclonal -p21 antibody (Pharmingen, San Diego, CA) (1:1000 dilution). A peroxidase-labeled, goat -rabbit antiserum (1:3000 dilution) (GibcoBRL) was used in conjunction with the ECL chemiluminescence system (Amersham) for detection of bound primary antibody. For detection of p21/PCNA complexes in VSMCs, quiescent (G0 + G1) primary rat aortic VSMCs were infected for 24 hours with 40 PFU per cell of AdlacZ or Adp21, stimulated to proliferate by incubation for 24 hours in medium containing 10% FCS, and cell lysates were prepared in Tween lysis buffer (50 mM Hepes, pH=7.5, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween-20) (1.5 X 10^6 cells in 1 ml of lysis buffer). Cell lysates were pre-cleared by incubation at 4°C for 1 hr with 100 µl of protein A sepharose (Pharmacia, Piscataway, NJ).

For immunoprecipitation experiments, 100 µl of protein A sepharose beads (Pharmacia) were mixed with 1µl of rabbit polyclonal -p21 antibody (Pharmingen) and incubated at 4°C for 60 minutes. The

resulting beads were washed 3 times in Tween lysis buffer, added to pre-cleared infected cell lysates containing 700 µg of protein as determined with a commercially available kit (Pierce, Rockford, IL) and incubated for 60 minutes at 4°C with gentle rocking. The beads were washed 3 times with Tween lysis buffer and the immunoprecipitated proteins were released by boiling in SDS-PAGE loading buffer, fractionated by electrophoresis in 15% denaturing SDS polyacrylamide gels and subjected to immunoblot analyses using a polyclonal rabbit -p21 antibody (1:1000 dilution) (Pharmingen) or a mouse -PCNA mAb (1:100 dilution) (Santa Cruz, Santa Cruz, CA) as described above.

For assessment of p21 expression in adenovirus-infected rat carotid arteries, arterial segments were harvested 4 days following balloon angioplasty and adenovirus infection. Two arterial segments infected with Adp21 and 2 segments infected with AdlacZ (60 mg total wet weight for each pair) were immediately placed in 200 µl of ice-cold homogenization buffer (100 mM NaCl, 1 mM EDTA and 20 mM Tris (pH 8.0), 1 mM iodoacetamide, 0.23 mM PMSF, 77 µM aprotinin, 1 µM leupeptin, 0.7 µM pepstatin A, and 77 µM benzamidine). Following brief homogenization with a polytron homogenizer (5 seconds X 3) on ice, 200 µl of 2X SDS-PAGE loading buffer (37) was added to each sample. 200 µg of total protein from each sample was subjected to immunoblot analysis as described above using the -p21 polyclonal antibody.

For assessment of Rb expression, passage 3 VSMCs cells were incubated in serum free medium for 96 hours, infected with adenovirus as described above and, 24 hours after infection, were stimulated to proliferate by incubation in growth medium. Cell lysates were prepared and protein corresponding to 5 X 10^5 cells from each sample was fractionated by electrophoresis in 7.5% SDS polyacrylamide gels and subjected to immunoblot analysis using an -Rb monoclonal antibody (1:200 dilution) (Pharmingen) and a peroxidase-labeled, goat -mouse IgG antiserum (1:2000 dilution) (Gibco/BRL) in conjunction with ECL chemiluminescence system (Amersham).

Arteries infected with Adp21 expressed markedly elevated levels of p21 as compared to the AdlacZ-infected arteries. Immunoblots from the AdlacZ- and Adp21-infected arteries contained equivalent levels of a second band of slower mobility. This band did not represent native rat p21 because native rat p21 migrates with identical mobility to human p21 in our SDS-PAGE conditions. However, it may represent a denatured form of rat p21 or, alternatively non-specific binding of the antibody reagents used in these immunoblots.

F. Histological Assessment of Restenosis

Rat carotid arteries were harvested 20 days after balloon injury and adenovirus infection. 5 μm sections from paraffin-embedded arteries were stained with hematoxylin and eosin and the neointimal and medial boundaries were determined on coded slides by an investigator blinded to the experimental conditions. Areas and ratios were determined by digital planimetry of tissue sections using the Image Pro-Plus image analysis system (Fryer Co, Chicago, IL). The I/M ratios were measured from 6 sections of each artery subjected to balloon angioplasty and adenovirus infection. The mean of these determinations was used to calculate the I/M cross-sectional ratios for each animal.

In a second series of experiments, rat carotid arteries were subjected to balloon angioplasty and immediately infected with 2×10^9 PFU of either Adp21 or AdlacZ. Arteries were harvested 20 days after balloon injury and restenosis, as determined by the neointima/media (I/M) area ratio, was assessed by digital planimetry. Carotid arteries from the AdlacZ-infected control arteries displayed an I/M ratio of 1.01 0.04 (n=5). In contrast, infection with Adp21 resulted in an I/M ratio of 0.54 0.11 (n=6), a 46% reduction as compared to the AdlacZ control group ($P < 0.001$). Thus, localized infection with a replication-defective adenovirus encoding human p21 at the time of arterial injury resulted in a significant reduction in neointima formation in the rat carotid artery model of VSMC proliferation and restenosis.

Several experiments were performed to assess the safety of localized arterial infection with Adp21. First, necropsies performed by an independent pathologist did not reveal any evidence of gross or microscopic tissue pathology in the Adp21-infected rats. Moreover, serum electrolytes, renal and liver function tests, complete blood counts and clotting parameters were all normal 20 days after balloon angioplasty and Adp21 infection. Finally, there was no evidence of localized arterial inflammation, necrosis or aneurysm formation in the Adp21-infected arteries 20 days after balloon angioplasty.

EXAMPLE 6: Clinical Uses of a Process of the Present Invention

Coronary artery disease (CAD) due to atherosclerosis is the leading cause of morbidity and mortality in the United States. PTCA remains a mainstay of therapy for symptomatic CAD, with more than 400,000 procedures expected in 1995 in the United States alone. While the procedure is initially successful in relieving arterial stenoses in the vast majority of patients, clinically significant restenosis continues to complicate the procedure in up to 40% of cases.

Numerous pharmacologic strategies including antiplatelet agents, anticoagulants, angiotensin-converting enzyme inhibitors and cytotoxic agents have failed to significantly reduce the rate of restenosis following PTCA. Recently, a number of gene based therapies for restenosis have been described. Adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene (HSV-tk) followed by the systemic administration of ganciclovir has been shown to inhibit restenosis in both the rat and pig models of arterial injury.

Although effective, there are several disadvantages of this cytotoxic approach as compared to cytostatic gene therapy using p21. First HSV-tk therapyinvolves the induction of VSMC death which is associated with concomitant intravascular inflammation and the potential for medial necrosis and aneurysm formation. In addition, this approach requires systemic ganciclovir therapy, which has been

associated with neutropenia, thrombocytopenia, and ventricular arrhythmias in humans.

Biopolymer-mediated delivery of antisense oligonucleotides directed to c-myb or PCNA and cdc2, have also both been reported to inhibit neointimal hyperplasia in the rat carotid artery injury model. However, recent studies have suggested that antisense oligonucleotides may cause degradation of multiple RNA species and have important non-specific effects on intracellular and cell surface proteins. In addition, antisense oligonucleotides may be subject to significant batch-to-batch variability and to date it is been impossible to efficiently deliver antisense oligonucleotides to the vascular wall using non-viral, catheter mediated approaches. Finally, adenovirus mediated over-expression of a non-phosphorylatable constitutively active form of Rb has also been shown to significantly reduce restenosis in both the rat carotid and pig iliofemoral artery models of balloon angioplasty.

A cytostatic gene therapy approach using catheter-mediated delivery of Adp21 as described herein is a clinically applicable, effective and non-toxic treatment for vascular proliferative disorders. The degree of inhibition of restenosis achieved by Adp21 gene transfer in the present studies (46%) was comparable to that achieved with over-expression of HAΔRb, as well as with HSV-tk gene transfer with systemic ganciclovir therapy, and the intraluminal delivery of antisense oligonucleotides. Furthermore, administration of Adp21 was not associated with significant inflammatory responses and clinical toxicity in the present study.

From a clinical standpoint, the fact that Adp21 appears to inhibit two proliferative pathways (cyclin/CDKs and PCNA) makes it a more potent cytostatic agent than AdHAΔRb. On the other hand, the more pleiotropic activities of Adp21 may result in more undesirable side effects *in vivo*. Co-administration of Adp21 and AdHAΔRb can be more efficacious than the administration of either virus alone. Finally, because cyclin/CDK kinases are important regulators of proliferation in a wide variety of cell types, adenovirus mediated over-expression of

p21 is likely useful for the treatment of other human diseases associated with deregulated cell proliferation. Similarly, the finding that over-expression of both p21 and Rb can be used to provide effective cytostatic gene therapy suggests that other cell cycle regulatory molecules such as p15 or p27, either alone, or in combination likely represent useful cytostatic gene therapy reagents.

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations can be applied to the composition, process and in the steps or in the sequence of steps of the process described herein without departing from the concept, spirit and true scope of the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Leiden, Jeffrey M.

Barr, Eliav

(ii) TITLE OF INVENTION: A Process of Inhibiting Non-Neoplastic Pathological Cell Proliferation

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

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(viii) ATTORNEY/AGENT INFORMATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTCCCG GGGAATTCAC CATGGGGTAC CCATACGATG TTCCAGATTA CG 52

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATAGCATTAT CAACCTTGGT ACTGG 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTGACGAGGC CCAGAGCAAG AG 22

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGGGGCCGGA CTCATCGTAC TC 22

WHAT IS CLAIMED IS:

1. A process of inhibiting non-neoplastic pathological cell proliferation of cells in vivo comprising increasing the level of an inhibitory cell cycle regulatory protein in the cells.

2. The process of claim 1 wherein the level of the inhibitory cell cycle regulatory protein is increased by increasing the expression of the protein in the cells.

3. The process of claim 2 wherein the cells are transformed with an expression vector that contains a polynucleotide that encodes the inhibitory cell cycle regulatory protein operatively linked to a promoter that drives expression of the vector in the cell.

4. The process of claim 3 wherein the expression vector is a replication-defective adenoviral vector.

5. The process of claim 1 wherein the inhibitory cell cycle regulatory protein is p21 or p53.

6. The process of claim 1 wherein the inhibitory cell cycle regulatory protein is a dominant-negative cell cycle regulatory protein.

7. The process of claim 6 wherein the dominant-negative cell cycle regulatory protein is a non-phosphorylatable form of Rb-1.

8. The process of claim 1 wherein the cells are fibroblasts involved in keloid formation following surgery, prostate epithelial cells involved in benign prostatic hypertrophy, uterine smooth muscle and fibroblasts involved in uterine fibroids, colonic epithelial and connective tissue cells involved in benign colonic polyps, neurons involved in benign neuromas or skin epithelial cells involved in hyperkeratotic skin diseases.

9. The process of claim 1 wherein the cells are vascular smooth muscle cells.

10. The process of claim 9 wherein the vascular smooth muscle cells are located in an artery.

11. The process of claim 9 wherein the vascular smooth muscle cells are located in a vein.

12. The process of claim 10 wherein the pathological proliferation is proliferation following arterial injury.

13. The process of claim 12 wherein the arterial injury is arterial injury that accompanies primary pulmonary hypertension, accelerated atherosclerosis following heart transplantation or glomeruloproliferative disorders.

14. The process of claim 10 wherein the pathological proliferation is proliferation during restenosis.

15. The process of claim 14 wherein the restenosis is restenosis following balloon angioplasty of the coronary arteries, restenosis following balloon angioplasty of peripheral arteries, or restenosis of arterial-venous shunts in renal dialysis patients.

16. The process of claim 11 wherein the pathological proliferation is proliferation during restenosis.

17. The process of claim 16 wherein the restenosis is restenosis of a bypass graft in coronary artery bypass restenosis and occlusion or a bypass graft in peripheral arterial bypass graft stenosis.

18. The process of claim 3 wherein the cells are vascular smooth muscle cells and the expression vector is delivered into a blood vessel that contains the vascular smooth muscle cells in its wall.

19. A process of treating a vascular smooth muscle proliferative disorder in an animal in need of such treatment comprising administering to the animal an effective amount of an expression vector that contains a polynucleotide that encodes an inhibitory cell cycle regulatory protein operatively linked to

a promoter that drives expression of the vector in vascular smooth muscle.

20. The process of claim 19 wherein the expression vector is a replication-defective adenoviral vector.

21. The process of claim 19 wherein the expression vector is administered into a blood vessel of the animal.

22. The process of claim 19 wherein the inhibitory cell cycle regulatory protein is p21, p53 or a non-phosphorylatable form of Rb-1.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 95/15191

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 A61K48/00 C12N15/86 C07K14/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	67TH SCIENTIFIC SESSIONS OF THE AMERICAN HEART ASSOCIATION, DALLAS, TEXAS, USA, NOVEMBER 14-17, 1994. CIRCULATION 90 (4 PART.2). OCTOBER 1994. I90, SELTZER, J. ET AL. 'Inhibition of vascular smooth muscle cell proliferation in vitro and in vivo by a replication-defective adenovirus encoding a non-phosphorylatable retinoblastoma gene product.' see abstract --- -/--	1-4,6-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 12251 (BAYLOR COLLEGE MEDICINE) 24 June 1993 see page 7, line 4 - line 24	1-3,5,8
Y	see page 13, line 4 - page 14, line 2 see page 19, line 29 - page 21 see examples 4,8,9 see claims	4,9-22

Y	WO,A,94 11506 (ARCH DEV CORP) 26 May 1994 see the whole document	4,9-22

X	WO,A,91 15580 (RES DEV FOUNDATION) 17 October 1991 see page 2, line 23 - page 4, line 8 see page 5, line 20 - page 9, line 6 see page 17, line 9 - page 20, line 23 see claims	1-3,5-8

X	SCIENCE, (1993 MAY 28) 260 (5112) 1330-5, WANG, C. ET AL. 'Regulation of the Ets-related transcription factor Elf-1 by binding to the retinoblastoma protein.' cited in the application see page 1333, right column - page 1334	1-3,6,7

X	NATURE, vol. 366, 16 December 1993 LONDON GB, pages 701-704, XIONG, Y. ET AL. 'p21 is a universal inhibitor of cyclin kinases' see the whole document	1-3,5

X	MOLECULAR AND CELLULAR BIOLOGY, vol. 12, August 1992 WASHINGTON US, pages 3431-3438, HAMEL, P. ET AL. 'Transcriptional repression of the E2-containing promoters EIIaE, c-myc, and RB1 by the product of the RB1 gene' see page 3433	1-3,6,7

X	CELL, vol. 75, 19 November 1993 NA US, pages 805-816, HARPER, J. ET AL. 'The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases' see page 812, left column - right column	1-3,5

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, June 1994 WASHINGTON US, pages 5320-5324, SLEBOS, R. ET AL. 'p53-dependent G1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein' cited in the application see the whole document	1-22
A	SCIENCE, vol. 265, 5 August 1994 US, pages 781-784, OHNO, T. ET AL. 'Gene therapy for vascular smooth muscle cell proliferation after arterial injury' cited in the application see the whole document	4,9-22
A	WO,A,91 09114 (RES DEV FOUNDATION) 27 June 1991 see the whole document	1,6,7
O,P, X	CIRCULATION, (15 OCT 1995) VOL. 92, NO. 8, SUPP. S, PP. 3593, CHANG, M. ET AL. 'CYTOSTATIC GENE-THERAPY FOR RESTENOSIS USING ADENOVIRVS-MEDIATED OVEREXPRESSION OF THE CYCLIN/CDK INHIBITOR, P21' see abstract	1-5,8-22
P,X	CIRCULATION RESEARCH, vol. 77, no. 2, August 1995 pages 266-273, BENNETT, M. ET AL. 'Apoptosis of rat vascular smooth muscle cells is regulated by p53-dependent and -independent pathways' see the whole document	1-5,8-22
P,X	SCIENCE, (1995 JAN 27) 267 (5197) 518-22, CHANG, M. ET AL. 'Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product.' see the whole document	1-4,6,7

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US 95/15191

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1995 OCT 13) 215 (2) 446-51, KATAYOSE, D. ET AL. 'Consequences of p53 gene expression by adenovirus vector on cell cycle arrest and apoptosis in human aortic vascular smooth muscle cells.' see the whole document ---	1-5,9, 10,12, 17-22
T	JOURNAL OF CLINICAL INVESTIGATION, (1995 NOV) 96 (5) 2260-8, CHANG, M. ET AL. 'Adenovirus -mediated over-expression of the cyclin/cyclin-dependent kinase inhibitor, p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty.' see the whole document -----	1-5,8-22

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/15191

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-22
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although these claims are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. claims 1-5,8-22 (all partially): A process for inhibition of pathological proliferation of non-neoplastic cells by increasing the levels of p21 in the cells
2. claims 1-5,8-22 (all partially): Id. for p53
3. claims 1-4,6-22 (all partially): Id. for a dominant-negative non-phosphorylatable form of Rb-1

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [X] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Infor. ..on on patent family members

International application No
PCT/US 95/15191

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